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REVIEW

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Advancing *in vitro* spermatogenesis: a comparative review of static and dynamic culture systems for reproductive biology and infertility treatment

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Abstract

Infertility is an important issue among couples worldwide affecting an estimated 50–80 million people globally. The World Health Organization estimates that male factors are responsible for approximately 20–30% of all infertility cases. *In vitro* spermatogenesis (IVS) is the experimental approach that has been developed for mimicking seminiferous tubule-like functional structures *in vitro*. Cell culture is one of the most important techniques needed in biology-based science, and the standard methods are usually 2-dimensional (2D) cultures such as T-flasks, tissue culture well plates, Petri dishes and well plates designed for spheroids formation. The conventional 2D cell culture has several restrictions. Then 3-dimensional (3D) cultures were created, and these static culture mediums also had limitations. Considering some of the limitations of conventional culture, static culture environments have been changed to dynamic culture medium systems. Techniques such as microfluidics and bioreactors have been developed to achieve more physiologically relevant tissue substitutes and show good potential to provide an effective approach for IVS. This review offers a comprehensive comparison of static and dynamic culture methods for IVS, their fundamental principles, advantages and limitations, as well as their potential in facilitating reproductive biology and infertility treatment.

Keywords

In vitro spermatogenesis; Male infertility; Static culture; Three-dimensional culture; Dynamic culture; Microfluidics; Bioreactors

Avances en la espermatogénesis *in vitro*: una revisión comparativa de los sistemas de cultivo estáticos y dinámicos para la biología reproductiva y el tratamiento de la infertilidad

Resumen

La infertilidad es un problema importante entre las parejas en todo el mundo, que afecta a un estimado de 50 a 80 millones de personas a nivel global. La Organización Mundial de la Salud estima que los factores masculinos son responsables de aproximadamente el 20–30% de todos los casos de infertilidad. La espermatogénesis *in vitro* (EIV) es el enfoque experimental que se ha desarrollado para imitar estructuras funcionales similares a los túbulos seminíferos *in vitro*. El cultivo celular es una de las técnicas más importantes necesarias en las ciencias biológicas. Los métodos estándar suelen ser cultivos bidimensionales (2D), como matraces T, placas de cultivo tisular, placas de Petri y placas diseñadas para la formación de esferoides. El cultivo celular 2D convencional tiene varias limitaciones. Posteriormente, se crearon cultivos tridimensionales (3D), pero estos medios de cultivo estáticos también presentaban limitaciones. Considerando algunas de las limitaciones de los cultivos convencionales, los entornos de cultivo estáticos han evolucionado hacia sistemas de medios de cultivo dinámicos. Se han desarrollado técnicas como la microfluídica y los biorreactores para lograr sustitutos tisulares más fisiológicamente relevantes, mostrando un buen potencial para proporcionar un enfoque efectivo para la EIV. Esta revisión ofrece una comparación exhaustiva de los métodos de cultivo estáticos y dinámicos para la EIV, sus principios fundamentales, ventajas y limitaciones, así como su potencial en el avance de la biología reproductiva y el tratamiento de la infertilidad.

Palabras Clave

Espermatogénesis *in vitro*; Infertilidad masculina; Cultivo estático; Cultivo tridimensional; Cultivo dinámico; Microfluídica; Biorreactores

1. Introduction

A comprehensive literature search was conducted in the PubMed, Web of Science and Scopus databases with no date of publication limit. The search terms based on Medical Subject Headings (MeSH) terms were: "Spermatogenesis, Stem Cells, Spermatogonia, Tissue Engineering, Cell Culture Techniques, Infertility, Male, *In vitro* Techniques, Testis, Regenerative Medicine, Bioreactors, Microfluidics and Culture Media". The selection process was multistaged, with an initial screening based on relevant titles, followed by a secondary review from the abstracts (Fig. 1). The final stage involved a careful examination of the whole article, focusing on IVS related to male infertility. More emphasis was given to factors that would improve culture medium for the treatment of male infertility and IVS. This review encompassed a wide array of studies in the field. This review discussed a wide extent of research within the discipline, including both 2D and 3D culture systems, static and dynamic culture methods, and the use of microfluidic devices and bioreactors. Special emphasis was given to emerging technologies and their potential to push *in vitro* spermatogenesis into clinical application.

2. Methods

Infertility, a serious reproductive condition that affects 10 to 15% of couples globally, is defined as the failure of a couple to obtain pregnancy after one year of unprotected sexual intercourse [1]. Male infertility causes can include genetic disorders, problems following cancer therapy, and disturbance during spermatogenesis [2–4]. Genetic defects that lead to infertility [5] are mainly structural and numerical [6] chromosomal abnormalities and Y-chromosome deletions [7]. Furthermore, a growing proportion of men are experiencing infertility as a result of radiation and gonadotoxic treatment [8, 9]. Patients who get these therapies are unable to generate spermatozoon because they reduce the spermatogonia stem cell (SSC) pool [10]. There is also no direct clinical treatment for azoospermia [1]. Approximately, 1 in 100 adult males suffer from infertility due to azoospermia and its incidence is 10 to 15 percent in infertile men [1, 11].

The cyclical process of spermatogenesis creates spermatozoa cells in the seminiferous tubules (STs) of the testis. Spermatogonial stem cells (SSCs), which are primitive male germ cells, sustain a man's fertility throughout his reproductive life and are crucial to this process [12]. Previous research has suggested that it takes 74 days for SSCs to produce spermatozoa [13]. In the mitotic division of SSCs, spermatogonial clusters are connected by cytoplasmic bridges and produce two types of cells. Dark type A spermatogonia and light type A spermatogonia are the most undifferentiated stages of spermatogonia. The dark type A spermatogonia will replenish the stem cell pool, while light type A spermatogonia undergo further differentiation. These cells give rise to type B spermatogonia, which then differentiate into primary spermatocytes. These primary spermatocytes undergo meiosis I to form secondary spermatocytes, which then undergo meiosis II to produce haploid round spermatids. The round spermatids further differentiate through spermiogenesis to form mature spermatozoa [14] (Fig. 2).

Currently, novel methods such as cryopreservation, SSC autotransplantation (SSCT) [15], and SSCs *in vitro* cell culture [16] have been developed to address male infertility issues

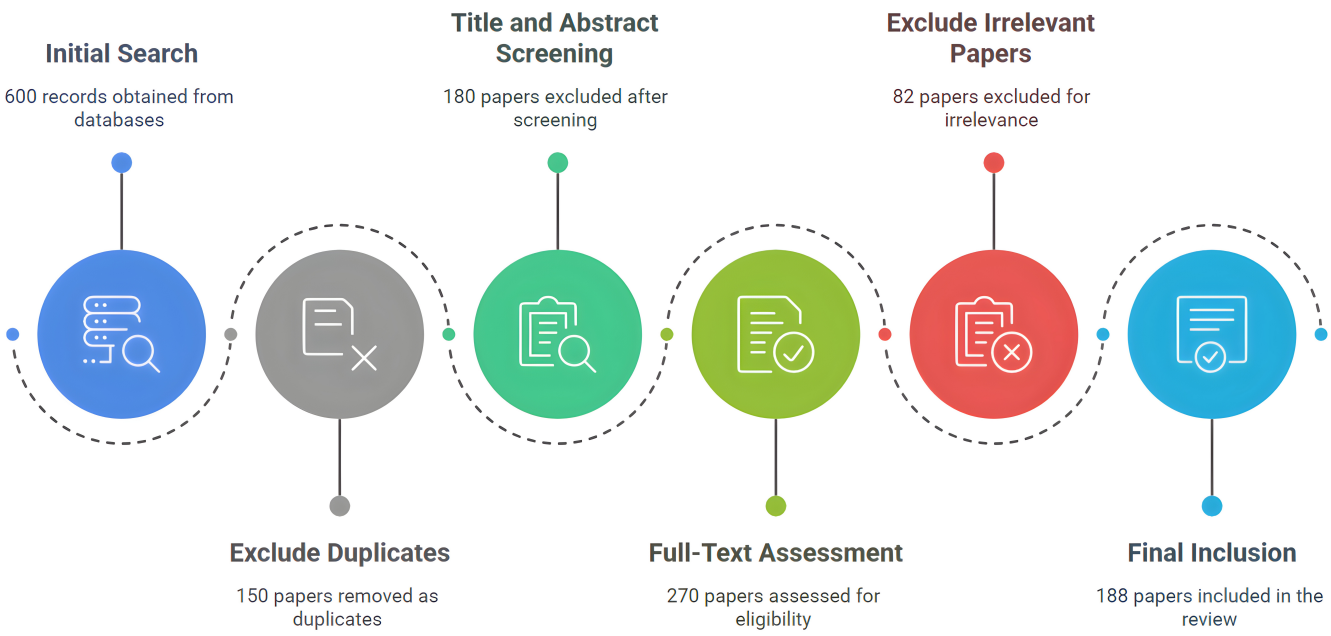

FIGURE 1. Flow diagram of literature search and study selection. Flowchart of the process of systematic literature search on PubMed, Web of Science and Scopus databases. 600 records were obtained on initial search. 150 papers were then excluded as duplicates. Then 180 papers were excluded after title and abstract screening. 270 full-text papers were assessed for eligibility. Then 82 full-text papers were excluded (due to reasons of irrelevant methodology, non-English papers). Finally, 188 papers were included in the review.

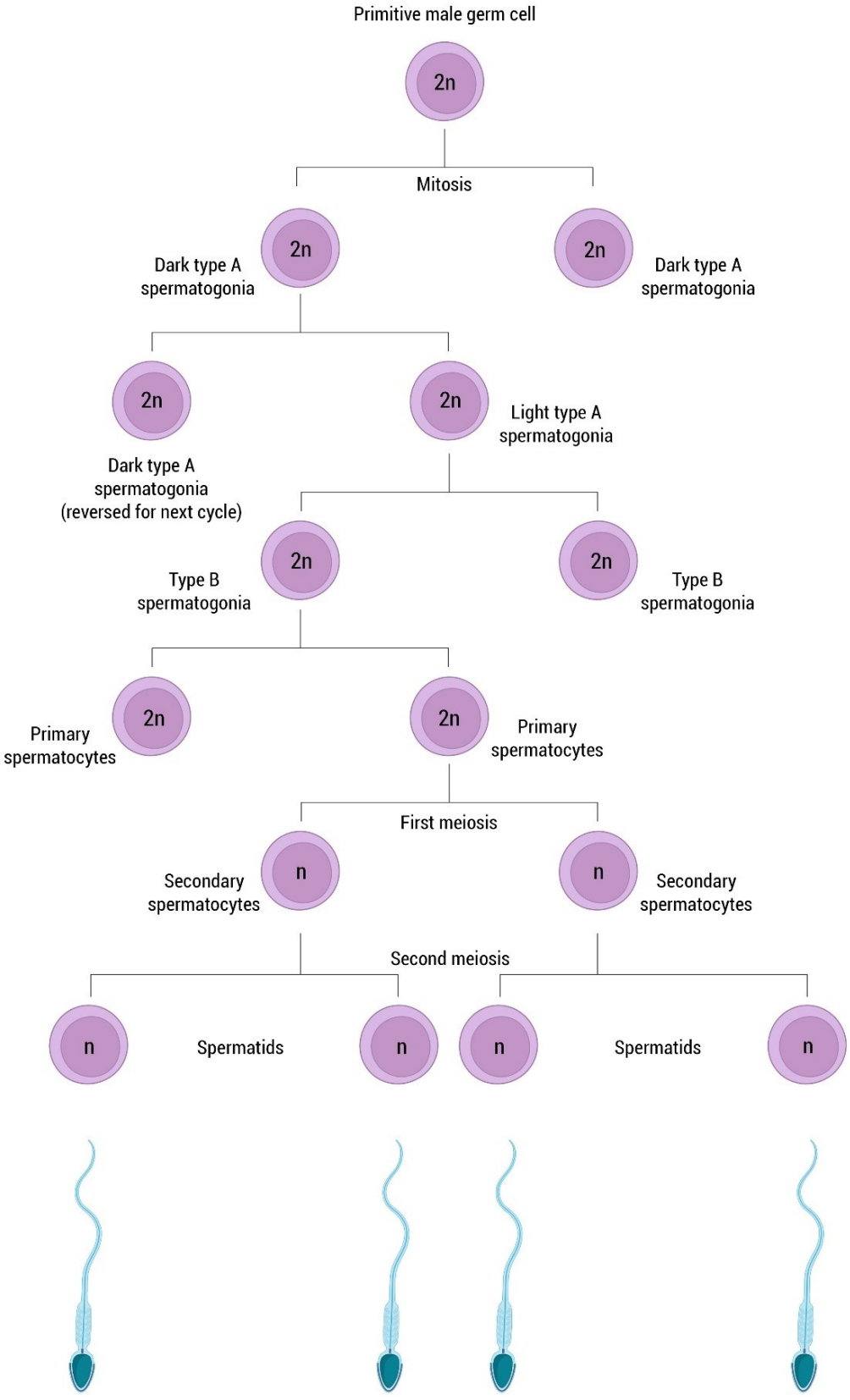

FIGURE 2. Spermatogenesis: development of sperm from primitive male germ cells to spermatids. The figure illustrates the principal stages of spermatogenesis, beginning with a primordial germ cell (spermatogonium), which undergoes mitosis to form primary spermatocytes. They divide by meiosis I to yield secondary spermatocytes, and by meiosis II to form spermatids. The illustration highlights the transition from diploid germ cells to haploid spermatids, which develop into mature spermatozoa.

[17]. Many of these infertile individuals no longer have SSCs or have lost them as a result of radiation and chemotherapy treatments, and a decline in the number of SSCs results in impaired spermatozoon production in these patients. Despite this, SSCs still possess the ability to self-renew and differentiate [18]. Yet, current treatments for such disorders remain invasive, expensive and have a low success rate [19].

Organ culture is the culture of whole organs or organ pieces in laboratory conditions, maintaining their 3D organization, cell-cell, and cell-extracellular matrix (ECM) interactions. Organ culture in testicular tissue allows for the culture of entire tissue or organ pieces instead of isolated cells, which could be effective for the study of IVS [20]. Although organ culture methods have been successfully used to investigate IVS in the small animal model, their application in large animal models or human is limited by challenges in maintaining tissue viability and replicating physiological conditions for long periods of time [21, 22].

Furthermore, testicular organ culture systems are often static, which limits their ability to accurately mimic the dynamic processes of testicular spermatogenesis and morphogenesis [20]. These limitations highlight the need for more advanced culture systems, such as dynamic or perfusion-based methods, to better mimic *in vivo* conditions and facilitate extensive research on testicular function and development [19, 23] (Fig. 3).

According to 2D systems, the main platform for studying male reproductive biology is the IVS of stem cells on plastic tissue culture plates. This method has several potential uses in reproductive research and medicine since it is inexpensive and simple [19, 23]. However, these 2D cultures could not simulate the cell architecture and appropriate biological cell-cell interactions. Therefore, a robust model that can simulate the complex cell-cell communication of the testis is needed (Fig. 3).

3D cell culture models can serve as an intermediate platform between 2D culture systems and animal models and mimic *in vivo* situations as much as possible [24]. In contrast to previous 2D cell culture techniques, 3D cell culture was created because of novel features. However, several problems with 3D static culture models usually exist, including poor homogenization [24], poor cell survival, poor dispersion and integration, and the formation of a necrotic core in the produced testicular tissue [25, 26] (Fig. 3).

It was time to modify the culture system in order to increase the efficiency of IVS. Therefore, the microfluidic method was employed, offering tissue conditions similar to those seen *in vivo* [27]. A chamber is designed for the tissue and a channel for the medium flow, so that the nutrients and waste products are exchanged with a molecular diffusion mechanism like what happens in the body [28]. The results showed maintenance of mouse testis tissue for 3 months and the production of spermatids and sperm, of which the ability of the resulting sperms to produce offspring was approved via micro-insemination [29]. These achievements in the microfluidic system, particularly the capacity to culture tissue for the long term, have motivated other studies to achieve IVS in human testicular tissue (Fig. 3).

Advanced 3D methods, such as bioreactors, have recently been created to cultivate testis tissue to mimic *in vivo*-like circumstances [30, 31]. Bioreactors are important devices that not only support and guide the development of *in vitro* body tissues

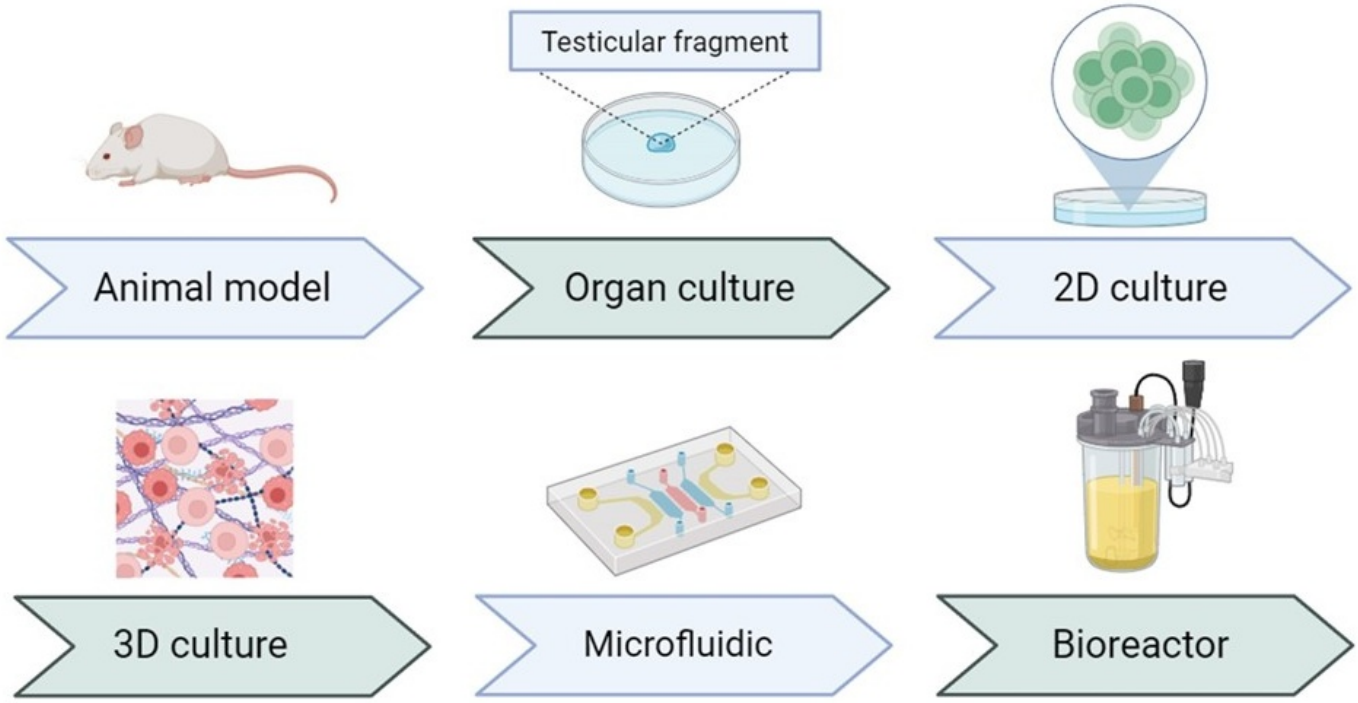

FIGURE 3. Evolution of culture methods in spermatogenesis studies. The historical progression of culture techniques used in spermatogenesis studies, from static organ culture methods to dynamic culture medium systems, with the use of bioreactors and microfluidic devices. 2D: 2-dimensional; 3D: 3-dimensional.

but also act as devices for cellular and biochemical assays [32]. Static culture limitations can be reduced or eliminated with bioreactors. In addition, a variety of bioreactor designs have been created that yield better results than the traditional two-dimensional cell culture [23, 30, 31]. Fig. 3 summarizes the historical progression of spermatogenesis research, highlighting key advancements in the development of testicular-like environments.

3. Testicular tissue culture

Testicular organ or tissue culture systems are valuable models for the study of spermatogenesis and testicular function since they allow researchers to control the microenvironment, including paracrine communication and growth factors (GFs), in a laboratory environment [33]. They offer the possibility of studying key regulatory mechanisms of spermatogenesis by controlling certain parameters through which the process is made up [33, 34]. The origins of IVS are connected with the development of organ culture techniques, dating back to nearly a century ago. Initial attempts at IVS relied heavily on organ culture techniques, particularly in the 1960s and 1970s when testicular fragments were cultivated in culture to observe spermatogenesis. But these early systems were under severe restrictions, since they were not able to maintain spermatogenesis beyond the pachytene stage of meiosis [35, 36]. Despite such restrictions, organ culture methods provided the foundation for the modern advancements *in vitro* maturation of spermatozoa, as described in the historical background presented by Sá *et al*. [36] (2007). Recent studies have highlighted the need for more sophisticated systems of culture that better mimic the dynamic physiological environment of the testis, particularly for applications in human and large animal models [22, 37] (Fig. 4).

3.1 Animal studies

Researchers have long taken an interest in a number of factors that contribute to testicular tissue culture, such as culture medium, temperature, oxygen and different culture systems, using animal models. Martinovitch *et al*. [38] (1938) were among the first to study, reporting on spermatogenesis development to the mid-pachytene stage in immature mouse testis tissue cultures of newborn mice. The culture medium used was fowl plasma and embryo extract [38]. In 1959, Trowell *et al*. [39] developed a novel gas-liquid interphase method, and that was the gold standard for organ culture. Organ pieces were in this system placed on a thin layer of agar over an underlying metal grid with holes, and the agar was moistened with culture medium to supply oxygen and nutrients. Although they were unable to induce spermatogenesis beyond

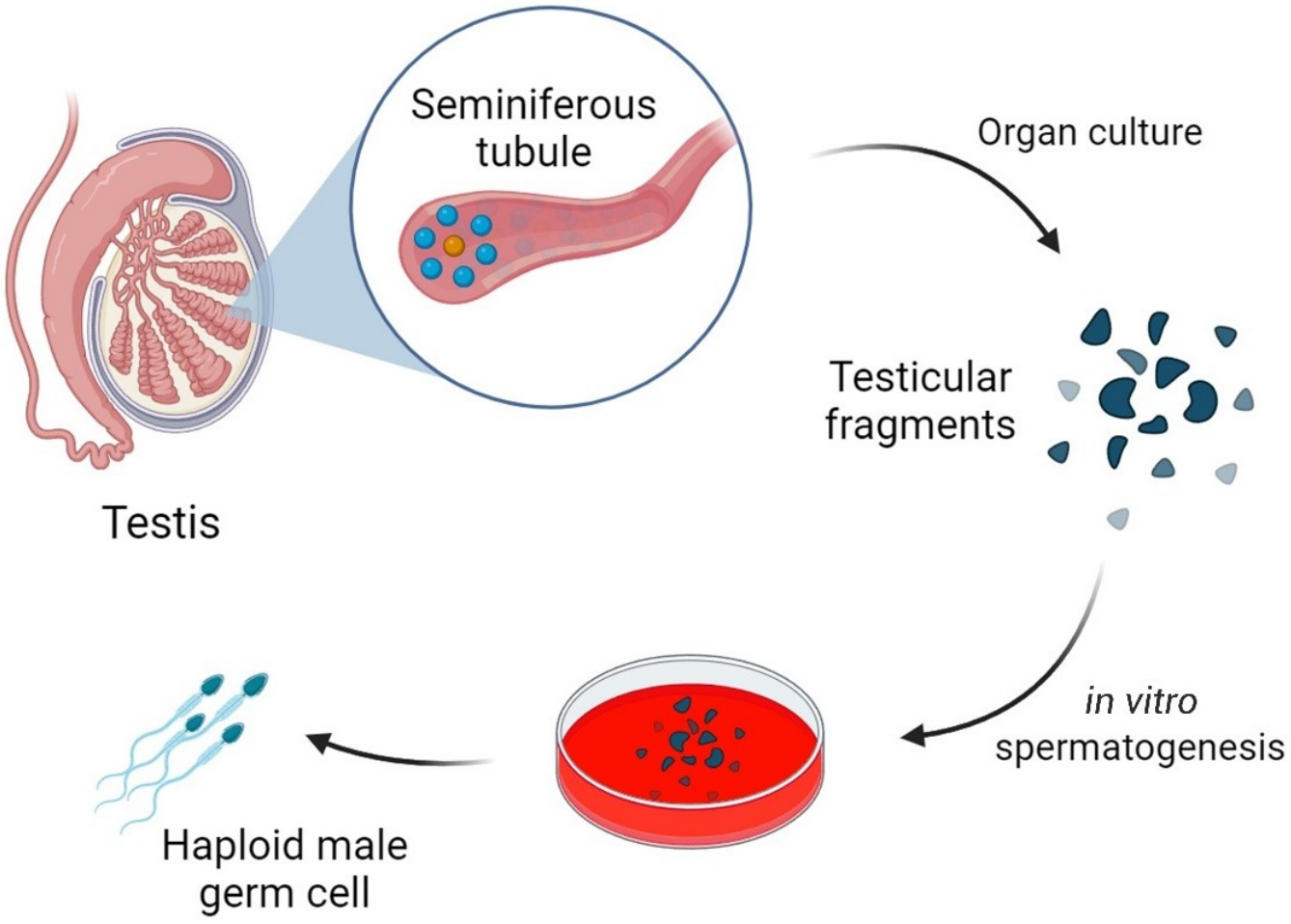

FIGURE 4. Testicular tissue culture method for IVS. Use of testicular tissue in static culture medium to study the process of spermatogenesis. This setup allows researchers to observe and analyze the stages of sperm development outside the body.

the pachytene phase, this work set the stage for subsequent experiments on the maximization of culture media [39]. One of the more complete early experiments was conducted by Steinberger *et al*. [40] (1964), who cultured testis tissue from numerous animal species including rats and measured several conditions including culture medium constituents, pH, temperature, gas phase, and the effect of vitamins and hormones. They had achieved rat testis tissue survival of 14-day-old to 14 months and followed development of spermatogenesis through zygotene to pachytene. Their results demonstrated the activity of Follicle-Stimulating Hormone (FSH) in Sertoli cell (SCs) differentiation, benefit of 95% air over 95% oxygen, and beneficial temperatures below 33 °C. They also proposed a chemically formulated medium promoting IVS in 4-day-old rat testis cultures to enable development from spermatogonia to the pachytene stage within 3 weeks [40–42]. Haneji *et al*. [43] (1982) demonstrated that FSH stimulates spermatogonial proliferation and germ cell differentiation in mouse testis cultures. They observed the development of type A spermatogonia to meiotic pachytene spermatocytes, highlighting the role of FSH in early spermatogenesis [43]. The study by Boitani *et al*. [44] (1993) used organ cultures of immature rat testicular tissue to investigate SSCs proliferation, focusing on the effects of GFs such as FSH. The results showed that FSH significantly stimulated spermatogonial proliferation *in vitro*, highlighting its role in the primary regulation of germ cells. Suzuki and Sato [45] (2003) achieved a milestone in recovering round spermatids from the culture of 5-day-old mouse testis tissue within 2 weeks. Their culture medium was similar to that of Steinberger *et al*. [42] once again reaffirming the contribution of optimized culture conditions towards the promotion of spermatogenesis [45]. A breakthrough in IVS was achieved when Sato *et al*. [46] (2011) produced offspring using *in vitro*-derived haploid germ cells. Using Minimum Essential Medium (MEM) culture medium with knockout serum replacement (KSR) or a lipid-rich bovine serum albumin supplement (AlbuMAX) as an alternative to fetal bovine serum (FBS), they were successful in achieving complete spermatogenesis in mouse testis cultures. This system was also effective in an infertile mutant mouse model, with potential as a therapy for spermatogenic defects caused by deficient factors. As a potential therapy for spermatogenic deficiencies caused by inadequate factors, this organ culture method was also successful in spermatogenesis in an infertile mutant mouse model.

3.2 Human studies

In addition to these findings, studies in human models have also been able to elucidate to some extent the important role of FSH and testosterone in proper testicular function during spermatogenesis and SCs function. For instance, Medrano *et al*. [47] (2016), studied the *in vitro* proliferative ability of human SSCs under culture conditions established for mouse SSCs. The strategies involved human SSC isolation and culture in a growth factor-supplemented medium with glial cell line-derived neurotrophic factor (GDNF), fibroblast growth factor (FGF-2), and other factors that have been used to sustain mouse SSC self-renewal. It was found that human SSCs appreciably did not proliferate under mouse-specific conditions, as observed with intrinsic species-specific variability in SSC niche requirement and signaling pathways. The study highlights the need to establish human-specific culture systems in an attempt to expand and explore human SSCs for fertility preservation and regenerative medicine applications [47]. Similarly, Sá *et al*. [22] (2012) demonstrated that T and FSH are essential to SCs proliferation as well as control of function within human testicular cultures, which supports the use of these hormones in optimizing culture conditions for IVS.

3.3 Tubule culture methods

Tubule culture, in which fragments of tubules instead of whole testis tissue are cultured, has been examined as an alternative to organ culture. This method eliminates interstitial cells and tissues and facilitates the evaluation of the contribution of parameters such as culture medium composition and environmental conditions [48]. There is no evidence, however, that tubule culture is superior to, or even as good as, organ culture for the eventual goal of IVS, *i.e.*, the formation of functional haploid germ cells [46].

Some studies have reported variation in the rates of success in culture of testis tissue among animal species, including between young and old members of the same species. For example, while mice models have shown consistent progress with IVS [46], other mammals, such as rats and non-human primates, have manifested lower efficiency in accomplishing total IVS [47, 49, 50]. The findings underscore the requirement for further work to establish better culture conditions to break through species-specific bottlenecks.

4. Two-dimensional (2D) culture

Organ culture was not possible anymore due to the persistent pachytene stage barrier during meiosis. Hence, researchers shifted their focus towards testis cell suspension culture to study SSC development and germ cell production (Fig. 5).

4.1 Animal studies

Steinberger *et al*. [51] (1966) originally cultured rat testicular cells but saw no differentiation of germ cells in this system. In 1983, breakthrough came when researchers successfully cultured enzymatically dissociated mouse testis cells which had reached the first prophase of meiosis. This work was the first to report on the crossing of germ cells of the pachytene barrier to the diplotene phase [52]. At the same time, Kierszenbaum and Tres (1983) demonstrated that the co-culture of SCs and germ cells was required for germ cell differentiation and survival, and similar findings were published by other groups [53]. Advances were made when 1c cells, which were isolated from enzymatically dispersed postpartum testicular rat cells, were cultured with type-1 collagen. These cells were differentiated into haploid cells, which was a major improvement in IVS [54].

The 15P-1 cell line, from transgenic mouse testicular cells that expressed the polyoma virus's large T protein, was used by Rassoulzadegan *et al*. [55] (1993). The round spermatids were produced by this cell line in 2–5 days of culture [55]. Similarly, Hofmann *et al*. [56] (1992) applied a germ cell immortalization approach, which, despite its limitations, produced a plateau

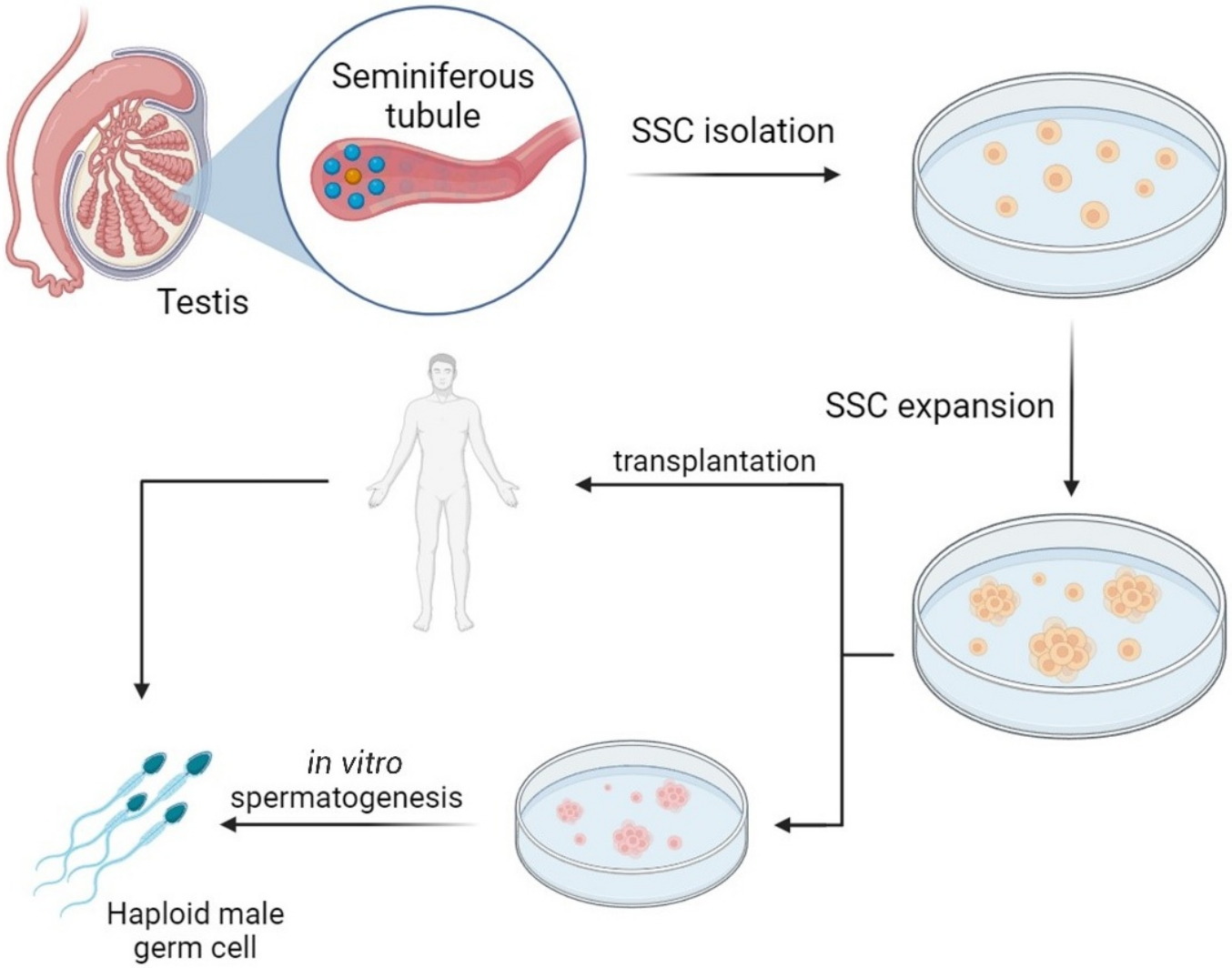

FIGURE 5. Two-dimensional culture method for IVS. Schematic diagram of a 2D culture system, including isolation of cells from testicular tissue and their growth on a culture dish, for use in autologous transplantation or IVS investigation. SSC: spermatogonial stem cell.

of haploid cells and acrosome granule production. The first-ever offspring derived from haploid cells developed *in vitro* was a landmark documented by Marh *et al.* [57] (2003). The co-culture experiment in this study entailed co-culturing SCs and 13–18-day-old mice germ cells that developed round spermatids with the ability to fertilize. This was subsequently done by Hasegawa *et al.* [58] (2005) [57]. The co-culture of germ cells and SCs of 13–18-day-old mice resulted in round spermatids with fertilization properties, which was later confirmed by other researchers like Hasegawa *et al.* [58].

4.2 Human studies

In clinical research, Tesarik *et al.* [59, 60] (1998 and 2000) demonstrated the initial 24 hours of spermatogenesis in obstructive and non-obstructive azoospermia patients. They co-cultured fresh and cryopreserved testicular biopsy tissues in FSH and testosterone-supplemented media, demonstrating partial advancement in spermatogenesis. Tanaka *et al.* [61] (2003) examined the use of Vero cells, a monkey kidney epithelial cell line, as a feeder layer to induce the completion of meiosis in primary spermatocytes of humans. Culture medium was also supplemented with FSH and testosterone, and the researchers assessed meiotic development by fluorescence in situ hybridization (FISH). The study also examined the practicability of resultant haploid cells for intracytoplasmic sperm injection (ICSI). Results demonstrated that, the Vero cell-based co-culture system favored the completion of human primary spermatocytes' meiosis and the formation of haploid cells. The formed haploid cells were successfully applied in ICSI for embryo production. Vero cells were seen to be a possible supportive feeder layer in IVS with cross-species compatibility in supporting human spermatogenic development [61]. Advances in culture medium composition and conditions over the past few years have improved the differentiation of germ cells towards the production of haploid cells [62]. For the first time, in 2003, male germ cell (round spermatid) generation *in vitro* was achieved, a significant advancement in human IVS [63].

The function of SSCs depends solely on a specialized niche composed of various cell types [64]. It has been demonstrated that multiple somatic cell populations are needed for the de-

velopment and maintenance of the SSC niche [65]. Besides, postnatal testis development involves maturation of somatic supporting cell populations that are responsible for creating a functional niche for SSCs [66]. SSC differentiation and undifferentiation are regulated by the niche, such as Sertoli cells (SCs), Leydig cells (LCs), peritubular myoid cells (PTMs) and endothelial cells (ECs) [67]. Each cell type helps in regulating self-renewing and differentiating processes of SSCs toward their maturation due to the secretion of GFs and maintenance of the optimal microenvironment. This means that elaboration of a cell type and interactions of an IVS of an appropriate phenotype will be vital in the next stage of optimization within human *in vitro* culture conditions, in a manner leading to better ways of therapeutic intervention in male infertility. Here, the role of somatic cells during spermatogenesis is briefly explained.

4.3 The contribution of somatic cells to spermatogenesis

4.3.1 Role of Sertoli cells (SCs) in spermatogenesis

Infertility is usually caused by SC defects, the sole somatic cell type in the testis critical for SSC development [68]. SCs secrete laminin, an ECM component of STs basal lamina that completes the stem cell niche by providing structural integrity and signaling factors necessary for SSC maintenance and regulation [65, 69]. This ECM protein not only keeps SSCs anchored in the niche but also controls their proliferation, differentiation, and survival through interactions with integrins and other cell surface receptors [69]. SCs also form tight junctions that comprise the blood-testis barrier, which segregates STs from blood vessels and provides physical support for germ cells. The barrier is essential for spermatogenesis and excludes toxic substances from the STs [70]. As has been shown in previous studies, the co-culture of germ cells and SCs *in vitro* promotes the development of germ cells, and it has been foundational strategy for IVS [21, 22, 71–74]. Kurek *et al*. [71] (2019) examined the mechanism of laminins in regulating stemness and germ cell development in human beings, focusing on their function in the stem cell niche. The study utilized *in vitro* co-culture systems of SCs and germ cells, and it was clear that SC-secreted laminins form a supportive microenvironment essential for germ cell progression and maintenance. Findings indicate that laminins are central to germ cell survival, proliferation and differentiation, including SSCs, through cell-ECM interaction mediation. Such findings highlight the importance of SCs and laminins in germ cell development and their potential applications in fertility research and regenerative medicine [71]. Sá *et al*. [21] (2008) examined SSC maintenance and differentiation mechanisms within the testicular niche. The study used *in vitro* co-culture systems of SSCs and SCs and took into account main signaling pathways, such GDNF, which plays a central role in SSC self-renewal. Findings showed that SCs facilitate SSC proliferation and inhibit differentiation via GDNF secretion and the deposition of a laminin-rich ECM. These results underscore the importance of SCs and niche microenvironment in SSC fate and offer hope for the preservation of male fertility. The study by Yokonishi *et al*. [68] (2020) demonstrates that SCs maintain the structural and functional integrity of the niche because their ablation leads to disruption in mouse SSC maintenance while their replacement rejuvenates SSC support and spermatogenesis.

Sousa *et al*. [74] (2002) investigated the developmental capability of human spermatogenic cells co-cultured with human SCs. FSH and testosterone were supplemented in culture media to mimic testicular niche-like physiological conditions. Human spermatogenic cells including spermatogonia, spermatocytes, and round spermatids were being studied in an attempt to establish their ability for meiotic development and spermiogenesis in the *in vitro* medium. The experiments demonstrated that SCs were of utmost significance in the maintenance of early stages of spermatogenesis. The co-culture system was supportive for the advancement of meiosis and for differentiation of round spermatids to elongated spermatids. However, spermatozoa maturation was not achieved, and other factors or specific culture conditions seem to be needed in order to complete spermiogenesis. These studies emphasize the central role that SCs play as a key cellular component of the niche, and their importance in the regulation of SSC self-renewal and differentiation. These findings are significant in uncovering the cellular mechanisms of spermatogenesis and potential means of fertility preservation [74].

4.3.2 Role of LCs, PTMs and ECs in spermatogenesis

Leydig cells (LCs) are extremely important in the process of spermatogenesis, as they secrete testosterone, a required androgen for male reproductive structure differentiation and development. LCs regulate spermatogenesis by generating growth factors (GFs) that supplement testicular activity and hormone activity such as testosterone, transforming growth factor-beta (TGF-β), interleukin 1α, insulin-like growth factor 1 (IGF1), inhibin, insulin-like peptide 3 (INSL3), colony-stimulating factor 1 (CSF1), thyroid hormones and estrogens [75, 76].

Peritubular myoid cells (PTMs) are seminiferous tubule (ST)-associated smooth muscle-like cells that provide structural support to the tubular wall as well as propulsion of non-motile sperm. They also release molecules such as leukemia inhibitory factor (LIF), GDNF, and Colony Stimulating Factor 1 that are crucial for SSC self-renewal and proliferation. Testosterone induces PTMs to secrete GDNF, whose direct action impacts SSC microenvironment and spermatogenesis [77–79].

ECs line blood vessels and regulate blood-vessel wall interactions. Within the testis, ECs secrete key GFs, including stromal cell-derived factor-1 (SDF-1), GDNF, FGF-2, macrophage inflammatory protein-2 (MIP-2), and insulin-like growth factor-binding protein-2 (IGFBP-2), necessary to maintain SSCs in culture [80–82].

The studies represent significant advancements toward comprehension of somatic cell and culture condition contributions to IVS. While there are many challenges to achieving the full maturation of spermatozoa, these findings provide a vital foundation for future work. By continued optimization of culture medium, enhancing somatic cell interaction and investigat-

ing other supportive factors, researchers may someday break through the barriers and achieve full IVS. However, 2D culture systems face limitations, such as the inability to fully replicate the complex three-dimensional architecture of the testicular niche. To overcome these challenges, the development of scaffold-based tissue engineering and cell culture techniques, which better mimic the physiological microenvironment, has emerged to advance IVS research [83].

5. Three-dimensional cultures

Many attempts in recent years to apply acceptable technologies capable of reproducing the spermatogenesis process *in vitro* have failed, and this has long been a barrier for researchers undertaking culture studies [84]. In the next step, these failed methods needed a deep evaluation to bring about improvements allowing them to be used further in research and to develop new applications and bring better results. The conventional 2D cell culture has several restrictions including the lack of ECM, cell-cell interactions and cell dynamics [85]. In a 2D cell culture model, cells grow on a flat surface in conditions that are different from those seen in the body, such as interactions between germ cells, stem cells and ECM, the effectiveness of the spermatogenesis process, and the gradient of GFs, cytokines and nutrients [86, 87]. To maintain, restore, or improve the function of a tissue or an organ, the conventional 2D cell culture has been changed from 2D pattern mode to 3D by the tissue engineering field to achieve more physiologically relevant tissue substitutes (Fig. 6).

For this purpose, reliable resources of tissues are required to construct or improve tissue function and in some cases, restore new tissues. To get over these restrictions, a cell delivery technology that possesses mechanical qualities that are comparable to those of the original tissue as well as biocompatibility and biodegradability is required [88]. A strategy to achieve better results of IVS is to use a 3D static culture medium [89]. Several studies have revealed that 3D static culture increases the proliferation and differentiation, migration, self-renewal, and viability of SSCs than conventional methods [78]. One of the first 3D cultures was performed in soft agar solution in the 1970s, and many advances have been made in the field of 3D culture to date [90]. In order to achieve the intended outcomes, scaffold forms and materials also play a crucial role. The possibility of negative impacts on reproduction is one element of biomaterials that is frequently disregarded. Therefore, choosing the most suitable material is

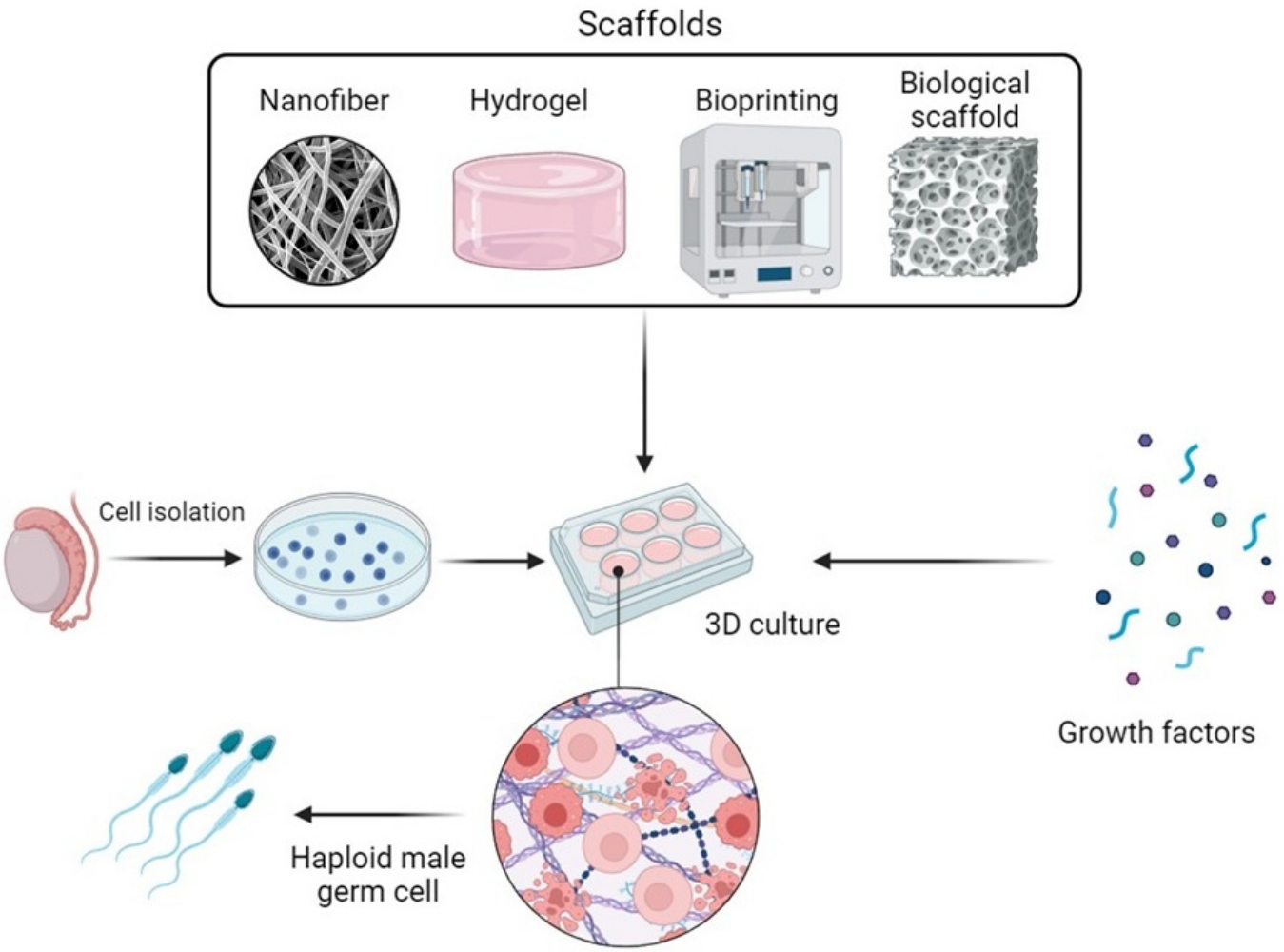

FIGURE 6. Three-dimensional culture method for IVS. Schematic illustration of the 3D culture system, illustrating the use of biocompatible scaffolds, testicular cells, and GFs to mimic the structural and functional microenvironment of the testicular niche. 3D: 3-dimensional.

important for creating a three-dimensional culture and many features have to be considered, for example, biodegradability, biocompatibility, permeability to oxygen, cost-effectiveness and mechanical properties (pore size, elasticity, stiffness and hydration degree), and cell adhesion [26, 91].

However, 3D static culture techniques frequently have a number of drawbacks, such as poor homogenization, poor cell survival, dispersion and integration, and the formation of a necrotic core in the produced tissues. Because they rely on static 3D cultures, 3D cell culture techniques usually lack precise control over gradients, are unable to match the multicellular complexity of tissues, and need medium exchange at discrete time intervals rather than continuously [26].

Owing to the limitations of organ culture, 2D culture and 3D culture there was a need to find alternative models, for mimicking seminiferous tubule-like functional structures *in vitro*, such as dynamic culture systems (Table 1, Ref. [25, 26, 78, 92–123]).

6. Microfluidic technique "labs-on-a-chip"

Several static culture techniques have been utilized widely for investigations of IVS. They have provided valuable data on the development of germ cells, testicular physiology and regulation of spermatogenic processes [122–125]. However, despite their utility, static cultures fail to closely reflect the dynamic conditions of the testicular microenvironment and cannot sustain long-term spermatogenesis or reflect accurately the conditions of *in vivo* [29, 126].

Notwithstanding its widespread usage, these methods have several drawbacks. One of the most important limitations of these methods is low efficacy in maintaining prolonged organ viability and function. A fundamental drawback in static cultures is that they do not possess any circulatory system that plays an imperative role in delivering new oxygen and nutrients, and in concomitantly eliminating the metabolized end-products [127]. This lack of dynamic exchange is known to establish gradients in nutrients, hypoxia and toxic byproduct accumulation, and finally undermines tissue culture health and function [87, 98].

To overcome these limitations, microfluidic technology or “lab-on-a-chip” platforms, have been developed as a new methodology (Fig. 7). These systems reproduce the physiological properties of *in vivo* systems by continuous fluid flow with full control over the delivery of nutrients, elimination of wastes and application of mechanical loading [128]. By reproducing salient aspects of the native tissue microenvironment, microfluidic platforms have demonstrated tremendous potential for enhancing the viability, functionality and relevance of *in vitro* organ cultures.

This technique has been used in clinical applications and diverse areas of research and could be applied as an alternative method to the treatment of male infertility [129]. Microfluidic devices would be useful tools for reducing either the costs or time of cell culture process and diagnostic systems. Microfluidics technique has become an important method to further increase the physiological relevance of 3D environment by enabling controlled co-cultures, spatial control of signaling gradients and perfusion, and spatial control over fluids in micrometer-sized channels [26, 130].

Lab-on-a-chip technology provides a groundbreaking approach to miniaturizing and automating biological and chemical processes. The devices are relatively simple in concept but fiendishly difficult to implement. A typical microfluidic device consists of three primary components: a housing chamber for cells or tissue, a reservoir for culture medium to contain the same, and a network of microchannels to connect these segments. The microchannels are typically open at one side in order to facilitate the collection of the medium when it is exiting the device, enabling continuous analysis or sampling [29, 87]. The main mechanism of the functioning of these devices is the diffusion of molecules through a semipermeable membrane that separates the cell or tissue from the culture medium. Thus, nutrition and waste products are being exchanged by passive diffusion so that the cells remain viable and functional for extended periods of time [87].

The membrane material is critical component in lab-on-a-chip device. The choice of microfluidic membrane significantly impacts the performance, durability and functionality of this device. In this study, we examined the benefits and limitations of common materials—glass, polydimethylsiloxane (PDMS), polycarbonate (PC), polyethylene terephthalate (PET), polyvinylidene fluoride (PVDF) and silicone—for microfluidic applications.

6.1 Glass

Glass is widely used in microfluidics due to its biocompatibility, chemical inertness and optical transparency, making it ideal for cell culture, diagnostic imaging, and biomedical assays [131–133]. Its high precision in microfabrication, thermal stability for sterilization, and smooth surfaces that minimize non-specific binding further enhance its utility. However, glass is brittle, expensive and challenging to scale for high-throughput or portable medical devices [134]. Additionally, its complex fabrication and bonding processes often limit its application in low-resource or point-of-care settings [135, 136] (Table 2, Ref. [127, 131–154]).

6.2 Polydimethylsiloxane (PDMS)

PDMS is a popular choice for microfluidic devices due to its biocompatibility, gas permeability and ease of fabrication. It is particularly well-suited for cell culture, organ-on-a-chip systems and prototyping. Its flexibility, cost-effectiveness, and ability to undergo surface modifications improve cell adhesion and reduce fouling [132, 136]. However, PDMS has notable drawbacks, including the absorption of small hydrophobic molecules, swelling in organic solvents, poor mechanical strength, and the potential degradation of surface modifications over time. These limitations restrict its use in harsh or long-term applications [127, 132, 136–138] (Table 2).

6.3 Polycarbonate (PC)

PC is valued for its cost-effectiveness, mechanical robustness and optical transparency, making it suitable for high-throughput fabrication, cell culture and fluorescence-based

TABLE 1. Comparison of organ culture, 2D culture, 3D culture and dynamic culture methods.

Culture Methods	Reference
Organ culture	
Advantage	
Maintains cell-cell contacts and signaling relationships and preserves tissue architecture	[92, 93]
Provide an opportunity to understand the biochemical and molecular functions of a tissue or organ	[93]
Disadvantage	
Maintaining organ cultures is difficult due to the different types of cells involved	[93, 94]
Difficulty in controlling experimental conditions	[93, 95]
Not suitable for biochemical and molecular analyses	[95]
2D culture	
Advantage	
2D cell cultures are typically easier to analyze, cell observation and measurement than some 3D cell culture systems	[25]
Learning the basics of 2D cell culture is easy	[25]
2D cell culture is well established and More cost effective	[25]
Disadvantage	
Lacks the effective and appropriate physiological microenvironment for the cells	[96]
Lack of the ECM, cell-cell interactions and cell dynamics	[96]
Lack of predictivity	[97]
Causing problems such as the production of toxic waste products, dead cells, depletion of nutrients and damage to the environment in which the cells are located	[98]
3D culture	
Advantage	
Developed to fill the gap between animal model and 2D cell culture (Reduces use of animal models)	[96]
Mimic *in vivo* situation as much as possible	[78, 99]
Possibility to grow concurrently two different cellular populations	
Cells tend to be more subjected to morphological and physiological changes to that in a 2D medium	[98, 99]
Scaffolds increase the proliferation and differentiation of spermatogenic germ cells than conventional methods	[78]
Disadvantage	
Formation of native gradient of autocrine/paracrine factor have not been considered	[100]
Development of a necrotic center of the formed tissue	[101]
Poor homogenization	[26]
Poor cell survival	[102]
Poor distribution and integration	[25, 103]
Challenges in microscopy and measurement in 3D cell cultures compared to 2D cell cultures	[25, 103, 104]

TABLE 1. Continued.

Culture Methods	Reference
Dynamic culture	
Advantage	
Promote organ culture	[95, 105]
Integration of fluid flow	[98]
Better simulation the physiological situations of testicular organ systems	[106–108]
The spatial fine control over fluids at micrometer scale	[109]
Parallelization of tissue collected from different individuals	[110, 111]
Provide the opportunity to observe the effect of periodic stimulation	[101]
Co-culture of several cells	[112]
Disadvantage	
Lack of proper access to cultured cell in 3D structures	[113, 114]
Lack of dynamic control and spatial presentation of different signal on 3D structures	[115, 116]
Need for more sampling for assays	[117, 118]
Complicated fabrication	[119]
Need for long-term studies to establish the genetic integrity of sperm	[120]
Need for standardization of protocols	[121]

2D: 2-dimensional; 3D: 3-dimensional; ECM: extracellular matrix.

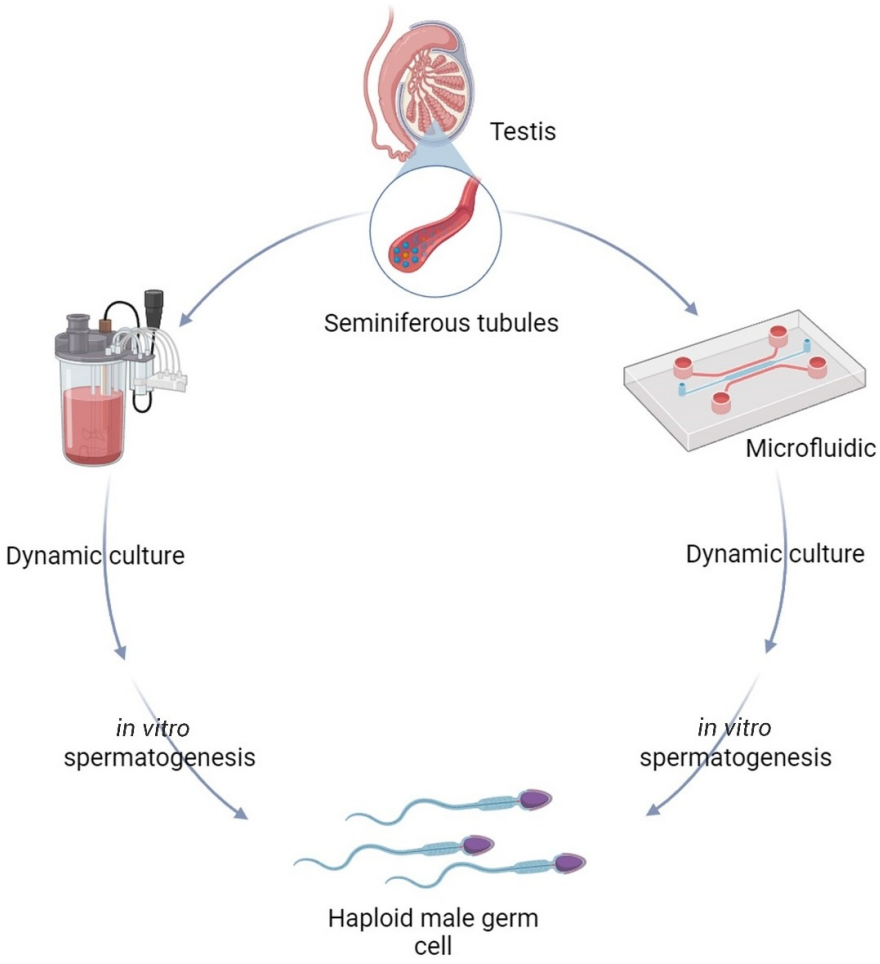

FIGURE 7. Utilization of microfluidics and bioreactors for IVS. Schematic illustration of dynamic culture system that employ microfluidics and bioreactors to simulate the physiological features of *in vivo* conditions, such as continuous fluid flow, precise delivery of nutrients, waste elimination and mechanical loading.

TABLE 2. The benefits and limitations of common microfluidic membrane—glass, PDMS, PC, PET, PVDF and silicone.

Material	Benefits	Limitations	Reference
Glass	Biocompatibility and chemical inertness, optical clarity, High precision, ideal for high-temperature sterilization, provides a smooth surface that reduces nonspecific binding of biomolecules in diagnostic assays, resistant to solvents and extreme pH conditions	Fragility, require complex surface modifications for specific biological interactions, High cost and time-consuming fabrication	[131–137, 152]
Polydimethylsiloxane (PDMS)	Flexible, easy to fabricate, biocompatible, gas-permeable, surface can be easily modified	PDMS can absorb small molecules, prone to swelling in organic solvents and has poor mechanical strength for high-pressure applications, poor chemical resistance, Surface modifications may degrade over time	[127, 132, 136–138]
Polycarbonate (PC)	High mechanical strength, chemical resistance, low cost, transparent, easy to mold, optically transparent and suitable for fluorescence-based detection	Require surface treatment for bonding, Limited flexibility, opaque, limited resistance to certain organic solvents and may degrade under UV exposure	[132, 137, 141, 142]
Polyethylene Terephthalate (PET)	Cost-effective, transparent and easy to mold, suitable for disposable devices in medical diagnostics	Limited thermal stability, requires surface modification, limited resistance to organic solvents and may degrade under UV exposure	[137, 141, 143–145]
PVDF	Chemical and thermal stability, durable, suitable for creating controlled wettability patterns	Difficult to bond, limited transparency, require surface modifications	[132, 141, 146, 147, 153, 154]
Silicon	High precision, thermal, chemical stability, mechanical stability	Expensive, rigid, opaque	[148–151]

PVDF: polyvinylidene fluoride; UV: ultraviolet.

detection. It is easy to machine and can be modified for hydrophilicity or hydrophobicity [132, 139]. However, PC has limited chemical resistance, may degrade under ultraviolet (UV) exposure, and often requires surface modifications for bonding or cell adhesion, complicating fabrication and long-term use [132, 137, 139–142] (Table 2).

6.4 Polyethylene terephthalate (PET)

PET is a lightweight, transparent and cost-effective material, making it ideal for disposable devices, rapid prototyping and electrochemical sensing. Its recyclability, compatibility with laser machining and hot embossing, and suitability for filtration and separation applications further enhance its appeal [132, 141]. However, PET has limited chemical resistance, may degrade under high temperatures or UV exposure, and often requires surface modifications for bonding or biological interactions, increasing fabrication complexity and cost [141, 143–145] (Table 2).

6.5 Polyvinylidene fluoride (PVDF)

PVDF is known for its chemical resistance, biocompatibility and piezoelectric properties, making it suitable for harsh environments, sensing applications and energy harvesting. It is mechanically robust and ideal for membrane-based and droplet-based microfluidic systems [141]. However, PVDF is less transparent, difficult to bond, and often requires specialized surface treatments or precise fabrication conditions, which can increase complexity and cost [132, 141, 146, 147] (Table 2).

6.6 Silicon

Silicon offers unparalleled precision and thermal stability, making it ideal for organ-on-chip systems, biosensors and precise cell manipulation. Its ability to integrate electronic and fluidic components enables advanced microfluidic applications [148–151]. However, silicon is brittle, expensive, and requires complex fabrication processes and cleanroom facilities, limiting its use in disposable or large-scale biomedical applications [148, 150, 151] (Table 2).

In more advanced designs, such as membrane-based multilayer devices (MM-D), the culture medium runs along the surface of the tissue chamber, while oxygen is supplied from beneath [155]. This two-way structure delivers the optimum nutrient supply and gas exchange with the physiological cell microenvironment very closely [156]. Monolayer devices employ a simpler design where oxygen moves vertically and culture medium moves horizontally. Both structures achieve comparable efficacy in cell function and viability with these variations [157].

The versatility of microfluidic devices is also demonstrated through their compatibility with various configurations and applications. Pumpless microfluidic devices, for instance, have achieved favorable performance comparable to pump-enabled devices. They deploy hydrostatic pressure from a reservoir of a medium to bring about fluid flow without requiring external pumps. A resistance circuit is incorporated to modulate the rate of flow such that the microenvironment can be perfectly controlled. It has been applied successfully to support spermatogenesis in mouse testis tissue for three months, demonstrating its promise for long-term tissue culture uses [87, 124].

One of the most powerful advantages of microfluidic techniques is precise spatial control over liquids at the micrometer scale, making it possible to simulate physiological conditions of target tissues. Microfluidic devices, for example, have been used to initiate and sustain spermatogenesis in mouse testis tissue for six months, yielding functional spermatids. These spermatids were then utilized for micro-insemination, and healthy offspring were delivered. This breakthrough emphasizes the potential of microfluidic devices for advancing reproductive biology and tissue engineering [87]. Microfluidic devices also demonstrated greater tissue survival and *ex vivo* gamete production compared with traditional methods. Microfluidic devices, under a controlled and dynamic microenvironment, enable researchers to study complex biological processes with new precision and reproducibility. This advantage has broad implications in drug discovery, regenerative medicine, and personalized medicine [35].

The assessment of gametes generated using microfluidic culture was carried out through micro-insemination in two distinct cell types that were cultivated for varying durations (41 and 184 days). In both groups, intracytoplasmic spermatozoon injections (ICSI) and round spermatid injections (ROSI) were effective and produced healthy progeny. These findings suggest that male gametes may be produced competently via microfluidic techniques [35]. Naeemi *et al*. [158] also explored the prospect of microfluidic chips for enhanced culture and assessment of SSCs. The findings show that microfluidic chips offer a more effective platform for maintaining SSC characteristics like viability, growth, and undifferentiated stem cell characteristics compared to conventional methods. This method highlights the potential of microfluidic technology to advance SSCs research and its application.

In addition to spermatogenesis, there are several other good properties provided by microfluidic devices. One is the ability to observe the tissue in real-time. The tissue is placed on a glass surface before loading into the device, allowing researchers to observe the process by a reverse microscope. This simple but really important feature allows for observing the growth of tissue and analyzing each step during the process which is helpful to fill the unknown gaps of spermatogenesis [157]. By gathering the medium that is already present in the device, the flowing medium provides an opportunity to quantify the quantity of testosterone generated by tissues. In contrast to *in vitro* methods, we may also increase the synthesis of testosterone by adding luteinizing hormone (LH) to the media [35, 87].

Another good quality that is represented by microfluidic devices is the parallelization of tissues collected from different individuals, enabling high-throughput analysis and comparative studies [159]. Microfluidics provides the opportunity to observe the effect of periodic stimulations. On the other hand, this technology allows researchers to investigate the effect of chemicals, drugs, and other types of material on gamete production and reproductive processes [160]. Other applications of microfluidics include cryopreservation, gamete management, embryo culture, and other assisted reproductive technologies [161, 162]. Microfluidics has a huge potential to improve the ARTs field yet there is a dire need for providing comprehensive data on human embryos to validate its efficacy. Furthermore, if the device is more cost-effective and user-friendly for laboratories, it would greatly increase their accessibility and acceptance in laboratory settings [162].

As previously mentioned, in order to effectively treat infertility using this technology and achieve more promising outcomes, it is crucial to pursue new and dynamic methods at a faster pace. It would be beneficial to explore the use of other mammalian tissues that closely resemble human tissue [163]. In the work of Kanbar *et al*. [164] (2022), compared the efficacy of microfluidic and static organotypic culture systems (agarose gel (AG), polytetrafluoroethylene membrane inserts (OT), and agarose gel with PDMS chambers (AGPC)) using prepubertal porcine testicular tissue was compared. The study aimed to identify the most effective system for supporting the *in vitro* maturation of immature testicular tissue (ITT) and evaluating tissue integrity, and germ cell survival in larger mammals. The results indicate the effectiveness of microfluidic systems and AGPC over the others. However, they cannot be related to human tissue because, as it appears, each kind of tissue needs its unique setup and cannot be expanded to others [164].

7. Bioreactors

As described in the microfluidic approach, the survival of the tissue is dependent on the rotation of nutrients and waste products produced by the tissue, which is accomplished *in vivo* by blood circulating in the capillaries surrounding the tissue. Diffusion is largely responsible for this activity [165]. Bioreactor systems are a significant step forward in IVS, providing a dynamic and highly controlled environment that optimizes the growth and development of germ cells [20]. The design of bioreactors and microfluidic systems has goals such as maintaining constant and optimal concentration of nutrients and gases, achieving uniform cell distribution, designing transparent chambers, carrying out mass transfer to the tissue, increasing tissue maturity by applying physical stimuli, and providing information about the formation of 3D tissue by attaching sensors [26]. Therefore, the right design may be used based on the goal of the study. Bioreactors and microfluidic methods have the capacity to give tissue a growing environment while also giving it an opportunity to exchange vital chemicals (Fig. 7). This is, in a sense, the advantage of dynamic culture tissue over statistic culture tissue, as the latter cannot have the entire tissue in contact with the medium; as a result, the tissue's ability to remove waste and take in oxygen and other metabolites will be limited, making it unable

to survive for an extended length of time [31, 165] (Table 1).

Also, 3D dynamic tissue culture provides the opportunity to maintain and develop different types of cells including germinal cells [35]. Conversely, one significant drawback of traditional bioreactors is that while they may support monocultures in three-dimensional cultures, they cannot support co-cultures. However,dual-chamber culturing techniques have been used to overcome this difficulty [26, 166]. Due to the different variety of these devices and their better understanding, they can be divided into 3 major groups: (A) in this group the medium is floated over the tissue or the cells, like hollow fiber bioreactors. (B) this group has heterogeneous mixing like stack plate reactors. The cells are grown on surfaces and stacked inside the vessels and the medium is circulated to them. (C) the cells are grown on microcarrier beads that are kept in suspension by some sort of mixing device, this group is called homogeneous mixing bioreactors. The two first groups are good choices for producing antibodies or types of cellular products. However, they are usually not efficient for cell sampling or analyzing the cell-cell interactions. Another point that is important to consider is the medium flow which should be in the optimum range otherwise the pores in fibers or plates will be plugged. The advantages that the 3rd group represents are the possibility of tissue modeling and a large surface for the cells to attach and grow which will take less space compared to the 2D tissue culture [167].

Amirkhani *et al*. [31] designed a perfusion bioreactor that successfully kept spermatogenesis from immature testis cells of the mouse for 8 weeks. The bioreactor used in this study was divided into three sections by three PDMS layers: the top, middle and bottom rings. The core of each of these rings has a cylinder cavity and a porose PVDF membrane. Two parts that are in the lower part are a medium flow chamber and a channel that unceasingly provides the culture medium from a perfusion pump that is located outside. The tissue was inserted in the middle section. By design, a syringe pump was used to remove the culture media from the entrance. Last but not least, the upper layer is the waste material chamber. Testicular tissue that has been grown on agarose gel and tissue grown in a bioreactor differ in that the tissue on the agarose gel has a necrotic core, but there is no proof that such components exist in a bioreactor. This indicates the value of culture flow in tissue growth. In this study, the higher efficiency of perfusion bioreactor over agarose gel culture was determined, and the bioreactor was also able to keep the testicular tissue differentiation up to the post-miotic stage. Some important subjects that need to be considered are the thickness of PDMS and pores, as well as flow rate which directly affects the tissue being cultured in a bioreactor [31]. Additionally, the PDMS layer lessens the quantity of oxygen in contact with the tissue, which lowers oxygen toxicity [29, 106, 157]. Furthermore, the proliferation of IVS is effectively aided by the 10% lower oxygen tension [168].

In an additional investigation, the application of stirred suspension bioreactors (SSBs) validates enhanced germ cell production and more effective handling of the material utilized in the cell environment. This type of bioreactor is good for handling large amounts of cells in nonrodent subjects [169]. SSBs consist of glass chambers that are sealed. In the central chamber, there is an impeller which is used to stir the cells and culture. Control over mixing speed and shear stress is made possible by this impeller. While the turbulence eddies are formed, the significance of mixing will become more apparent. For this reason, the cells are in aggregates and should maintain an optimal rotation speed to prevent their breakdown as well as keep the proper shear stress [170]. The chambers also have pores for medium and oxygen to enter and wastes to exit [30].

In another study, Zhang *et al*. [171] reported successful culturing of spermatogonia for 14 days using a dynamically stimulated microgravity rotating cell system. One of the flaws of rotating cell system is that scaling is hard and feeder cells are required. This problematic feature could be resolved by the use of SSBs [30]. In a study conducted by Sakib *et al*. [30], the aim was to create a dynamic culture medium system that allows the scalable expansion of porcine pre-pubertal spermatogonia using SSBs in a controlled environment. Perrard *et al*. [172] cultured the frozen or fresh human or rat STs using a hollow cylinder of chitosan bioreactor for 60 days. Spermatogenesis was entirely completed in this research, resulting in the full production of spermatozoa. This is the first instance of human spermatogenesis occurring entirely in a cultivated environment [172].

This method yields sufficient data for use in prepubertal cancer cases receiving chemotherapy and radiation treatment. Patients who have these cells preserved may become fertile in the future. This can be accomplished by amplifying a tiny percentage of immature testicular cells in a bioreactor, freezing the resultant spermatozoon for later use, and then repeating the process [172–177]. Eventually, bioreactors and microfluidics have produced substitutes that have high promise to offer an efficient method for IVS. These methods do, however, have several drawbacks, including the need for additional sampling for tests that still need to be worked out, access to cultured cells in 3D constructs, and a lack of control over the dynamics and spatial presentation of different signals over 3D constructions [113–116, 121].

8. The integration of bioreactors and microfluidics in IVS

The integration of microfluidic systems with bioreactor technology has immensely advanced IVS by developing conditions that closely approach natural physiological conditions. Bioreactors provide dynamic conditions, including mechanical stimulation and controlled oxygenation, necessary for maintaining testicular tissue and for germ cell growth. For example, work by Amirkhani *et al*. [31] has proven that miniaturized bioreactor platforms are able to maintain mouse testicular tissue and induce germ cell maturation over the long term. Similarly, work by Komeya *et al*. [35] demonstrates the ability of microfluidic platforms to maintain testicular tissue and yield viable spermatozoon with highly controlled management of nutrient supply as well as waste removal [35]. They complement each other to yield a hybrid system that more accurately replicates the testicular microenvironment, optimizing the success of IVS [178].

Microfluidic devices are ideally suited for manipulating fluid dynamics, nutrient supply and cell-cell interaction, which

are all critical in germ cell growth. To illustrate this, Shen *et al*. [179] demonstrated the application of microfluidic chips for human testicular tissue perfusion in order to allow extensive study of spermatogenesis under highly regulated conditions. Furthermore, Kashaninejad *et al*. [180] mentioned the capability of microfluidics to create intricate reproductive models such as testis-on-a-chip devices. Through these systems, researchers can investigate the intricate process of spermatogenesis accurately, giving clues about the determinants of germ cell development [108].

Combining bioreactors and microfluidics addresses the drawbacks of traditional static cultures by providing structural support and dynamic conditions. For instance, Horvath-Pereira *et al*. [181] pointed to biomaterials and bioreactors as having the potential to serve as a scaffold for testicular tissue, while microfluidics can facilitate improved oxygen and nutrient transfer. Such a synergistic model is also disclosed in another study, which demonstrated that coculture systems in bioreactors are capable of enhancing the function of testicular cells [182]. With the incorporation of microfluidics, such systems exhibit more accurate control of the microenvironment, leading to enhanced differentiation and maturation of germ cells [33].

Latest developments in tissue engineering have also expanded the capabilities of bioreactor-microfluidic systems in supporting IVS. For instance, Sabetkish *et al*. [183] investigated the use of decellularized testicular scaffolds as a natural bioreactor that can be integrated with microfluidic systems for the facilitation of cell seeding and delivery of nutrients. Another report [184] indicated the potential of 3D culture systems integrated with microfluidics for producing functional spermatids, which brings fresh hope to infertility treatment. These advances are a giant step towards the overcoming of male infertility and the improvement of reproductive science [185].

The ability to scale up and replicate repeatedly in bioreactor-microfluidic systems is critical for their application in clinical settings. Research has made it clear that these systems can effectively replicate complex physiological processes, a factor that is significant in the creation of effective treatments for infertility [186]. As an example, research has indicated the manner in which microfluidics enhances the precision and effectiveness of spermatozoon analysis and selection [187]. When combined with bioreactors, these kinds of systems are scalable to make larger volumes of viable spermatozoon and are therefore suitable for clinical application [180].

Despite this, challenges remain in the optimization of bioreactor-microfluidic systems for IVS. For example, long-term experiments must be carried out to ensure the genetic integrity and quality of the spermatozoon produced by these methods [122]. Standard protocols must also be established to ensure that there are reproducible and consistent results [121]. These challenges will be overcome by continued multidisciplinary efforts together with further advancement in tissue engineering and microfluidics [188].

9. Conclusions

The advances in cell culture methods, including 2D culture media, organ culture, and 3D culture systems, have significantly increased our ability to study and mimic physiological conditions *in vitro* substantially. The 2D culture media, while useful in basic cellular research, likely do not mimic the complex microenvironment of tissues. Organ culture, which maintains the architecture of tissue explants, provides a more realistic model but is limited by its short lifespan and lack of scalability. In contrast, 3D culture systems offer a more physiologically relevant environment by enabling cell-cell and cell-matrix interactions, thereby better replicating *in vivo* conditions. However, the transition from 2D to 3D cultures introduces challenges such as nutrient diffusion limitations and the need for dynamic culture conditions to maintain cell viability and function.

In order to surpass these challenges, bioreactors and microfluidic systems have been established as useful tools. Bioreactors create a dynamic condition via circulation of culture media, mechanical stimulation and optimum oxygenation, which are essential for the long-term survival and maturation of tissues. Bioreactors are excellently applicable for monocultures in 3D systems but are not very capable of accommodating co-cultures. Microfluidic devices, on the other hand, offer microscale control over fluid flow, nutrient gradients, and cell-to-cell interactions with high precision and therefore are ideally suited for creating controlled co-cultures and mimicking physiological signaling gradients. Also, the choice of membrane material for microfluidic devices depends on the specific requirements of the application, such as optical transparency, chemical resistance, mechanical strength, and scalability. Understanding these trade-offs is essential for designing effective and efficient microfluidic devices tailored to biomedical and biological research.

However, both bioreactors and microfluidics possess a share of limitations, such as the need for further sampling, limited access to cells in 3D constructs, and challenges in exerting control over dynamic and spatial signaling in 3D systems.

The integration of bioreactors and microfluidic systems is an excellent advancement in the area of reproductive biology and regenerative medicine. They are designed to provide a dynamic environment pertinent to physiology for long-term tissue maintenance, germ cell differentiation and spermatozoon production. By integrating these approaches, hybrid systems are created that more closely mimic the testicular microenvironment, making IVS more efficient and scalable.

Advances such as the use of decellularized testicular scaffolds, 3D culture systems and organ-on-a-chip platforms have also furthered the reach of these technologies. Such advances not only improve the physiological relevance of experimental models but also make clinical applications possible, for instance, the production of functional spermatozoa for the cure of infertility. However, challenges remain to be addressed, however, such as the need for long-term studies to establish the genetic integrity of sperm, standardization of protocols, and optimization of biomaterials for biocompatibility and function.

The future of research into male infertility and treatment lies in the ongoing optimization of bioengineered systems

that combine tissue engineering, microfluidics and bioreactor technologies. With facilitation of interdisciplinary synergy and correction of existing shortcomings, such systems hold promise for revolutionizing reproductive medicine, offering new hope to patients facing infertility and fostering greater insight into reproductive biology.

AVAILABILITY OF DATA AND MATERIALS

The data used and analyzed during the current study are included within the article. Further details can be provided by the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS

NR and JJ—contributed equally as co-first authors. NR, JJ and AA—wrote the manuscript draft. AG and ASH—were responsible for editing and revising the manuscript. MMMT and JJ—designed the figures. All authors reviewed and approved the final version of the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study did not involve human participants, human data or animal experiments.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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